(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
8 March 2001 (08.03.2001)

PCT

(10) International Publication Number
WO 01/16153 A1

(51) International Patent Classification[7]: C07H 21/02, 21/04

(21) International Application Number: PCT/US00/24213

(22) International Filing Date:
1 September 2000 (01.09.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/152,062 2 September 1999 (02.09.1999) US

(71) Applicant *(for all designated States except US)*: **UNIVERSITY OF MEDICINE AND DENTISTRY OF NEW JERSEY** [US/US]; 335 George Street, Suite 3200, New Brunswick, NJ 08903-2688 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **PESTKA, Sidney** [US/US]; 82 Brookside Terrace, North Caldwell, NJ 07006-4413 (US). **KOTENKO, Sergei, V.** [US/US]; 458 Andover Place, East Brunswick, NJ 08816 (US).

(74) Agents: **REED, Janet, E.** et al.; Centre Square West, 1500 Market Street, 38th Floor, Philadelphia, PA 19102 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *With international search report.*
— *Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/16153 A1

(54) Title: CYTOMEGALOVIRUS-ENCODED IL-10 HOMOLOG

(57) Abstract: Nucleic acids encoding a new cytomegalovirus-encoded homolog of interleukin 10 (IL-10) and methods of use thereof are disclosed herein. Also provided are pharmaceutical compositions comprising cmvIL-10 for the treatment of various pathological conditions.

CYTOMEGALOVIRUS-ENCODED IL-10 HOMOLOG

This application claims priority to U.S. Provisional Application No. 60/152,062, filed September 2, 1999, the entirety of which is incorporated by reference herein.

Pursuant to 35 U.S.C. §202(c), it is acknowledged that the U.S. Government has certain rights in the invention described herein, which was made in part with funds from the United States Public Health Services, Grant Nos. RO1-CA46465 and 1P30-CA72720, National Cancer Institute, Grant No. RO1-AI36450 and the National Institute of Allergy and Infectious Diseases, Grant No. RO1 AI43369.

FIELD OF THE INVENTION

This invention relates to the field of molecular biology, virology and immunology. In particular, the invention provides a novel cytomegalovirus-encoded homolog of interleukin 10 (IL-10) and its uses in IL-10-mediated therapies; in the development of therapies for cytomegalovirus-related diseases; and in the diagnosis of CMV infection and contamination of organ transplants.

BACKGROUND OF THE INVENTION

Various publications or patents are referenced in this application to describe the state of the art to which the invention pertains. Each of these publications or patents is incorporated in its entirety by reference herein.

Interleukin 10 (IL-10) is a pleiotropic, immunomodulatory cytokine produced by $CD4^+$ and $CD8^+$ T-

cells, monocytes/macrophages, keratinocytes and activated B-cells. Its expression is elevated in patients with a variety of peripheral blood or bone marrow-derived leukemias, certain B-cell, T-cell and nasal NK-cell lymphomas, as well as other hematopoietic and solid tumors.

IL-10 could contribute to tumor growth by two possible mechanisms. IL-10 appears to act as an autocrine growth factor for B-cell lymphomas. In addition, IL-10 selectively inhibits certain responses of the cell-mediated immune system. It blocks proinflammatory cytokine synthesis and suppresses the ability of macrophages to serve as antigen-presenting or costimulatory cells. Thus, IL-10 is a powerful anti-inflammatory agent and a potent immunosuppressor.

Viruses have developed elegant mechanisms to circumvent detection and destruction by the host immune system. One of these strategies to escape immune surveillance is to express immune modulators encode within viral genomes. Many viruses capture host genes and use them to their own advantage.

Several viruses have been found to exploit the tumor-supporting properties of IL-10 by adopting the strategy of using homologs of cellular cytokines or cytokine receptors to shield virus-infected cells from immune defenses and enhance virus survival in the host. The use of virus-encoded homologs of cellular proteins as a survival/defensive strategy is suggestive of an important role of these cellular compounds for *in vivo* host response to these viral invaders.

A number of herpes viruses produce homologs of IL-10. Epstein Barr virus (EBV)-encoded IL-10 was the first viral IL-10 (vIL-10) to be cloned. The ebvIL-10 shares many, but not all, of the biological activities of human or cellular IL-10, (referred to herein

interchangeably as cIL-10 or IL-10) and may play an important role in the host-virus interaction during EBV infection. In addition to EBV, another virus which can infect humans, the Orf poxvirus (OV), has its own IL-10 homolog (ovIL-10). Whether it is active on human cells remains to be shown.

The exact role of viral homologs of IL-10 in the viral life cycles, in immune evasion and/or in helping virus-infected cells to survive immune surveillance is not understood. The identification and further analysis of other viral homologs of IL-10 would facilitate the elucidation of the role of viral IL-10 in the survival and propagation of such viruses.

Human cytomegalovirus (CMV) is a widespread herpes virus able to persist for decades in its host. CMV is the major cause of a variety of life-threatening diseases in immunocompromised individuals, such as transplant and AIDS patients, as well as a leading cause of congenital birth defects. CMV has been reported to be associated with a variety of human cancers, including cervical carcinoma, adenocarcinoma of the colon and prostate, and Kaposi's sarcoma. CMV is also associated with the development of atherosclerosis, restenosis after coronary angioplasty, chronic organ rejection in transplant recipients, and chronic graft-versus-host disease in bone marrow transplant recipients. The complete sequence of the genome of cytomegalovirus (CMV) strain AD169 was determined in 1990 (Chee et al., (1990) Curr. Top. Microbiol. Immunol. 154: 125-169), but the functions for many of the open reading frames are yet to be discovered. Specifically, the CMV genome has not been reported thus far to contain any sequences that encode an IL-10 homolog.

The identification of viral gene products homologous to cellular genes, and the elucidation of

their functions, not only improves our knowledge of virus-host interactions, but enhances our understanding of the regulation of normal immune mechanisms, because of the relationship to their cellular counterparts. Such genes may have many functional applications.

SUMMARY OF THE INVENTION

In accordance with the present invention, a new viral interleukin 10 (vIL-10) encoded by the open reading frame, UL111a in the CMV genome, and homologous to cellular IL-10 (cIL-10) has been identified. This cytomegalovirus-encoded interleukin, designated cmvIL-10, can bind to the human IL-10 receptor and can compete with human IL-10 for binding sites, despite the fact that these two proteins are only 27% identical. cmvIL-10 requires both subunits of the IL-10 receptor complex to induce signal transduction events and biological activities. Expression of cmvIL-10 in cells causes their malignant transformation and renders them able to form tumors. Thus, cmvIL-10 is a viral oncoprotein which may be a major factor responsible for driving cells to malignant transformation in certain CMV-associated cancers.

According to one aspect of the invention, an isolated nucleic acid molecule is provided, which comprises a nucleic acid segment spanning nucleotides 159675 through 160376 of the CMV genome and which encodes a cmvIL-10 protein that is encoded as a precursor polypeptide with a signal sequence. This sequence covers the entire cmvIL-10 coding region, composed of three exons; there are also two introns which are spliced out of the longer sequence to produce the complete and intact cmvIL-10 open reading frame. The exact nucleotide numbering is as follows: 159678 is the first nucleotide of the first ATG (Met) codon (of the signal sequence),

160364 is the last nucleotide in the TAG stop codon (GenBank Accession No: X17403).

In a preferred embodiment, the nucleic acid molecule is inserted in a heterologous vector. In a particularly preferred embodiment, the vector comprising the insert is plasmid pEF-SPFL-cmv_2. In a preferred embodiment, the nucleic acid molecule encodes SEQ ID NO:2 or a variant thereof described in greater detail below. In a particularly preferred embodiment, it comprises SEQ ID NO:1.

According to another aspect of the invention, an isolated protein is provided, which is a CMV-encoded cmvIL-10. The cmvIL-10 preferably is produced by expression of the protein encoded by a nucleic acid inserted into a heterologous vector. In a preferred embodiment, the protein comprises SEQ ID NO:2 or a variant thereof as described in greater detail below. It will be understood by those of skill in the art that the isolated mature cmvIL-10 will lack the signal sequence of the precursor polypeptide.

According to another aspect of the invention, a pharmaceutical formulation for treating patients having an IL-10-treatable disease is provided. The formulation comprises a cmvIL-10 protein, or a gene encoding a cmvIL-10 protein, in a pharmaceutically-acceptable medium.

According to another aspect of the invention, a method of treating patients having an IL-10-treatable pathological condition is provided. The method comprises administering to the patient an effective amount of the above-described pharmaceutical formulation which comprises the cmvIL-10.

According to another aspect of the invention, a method of treating a pathological condition associated with CMV infection is provided. The method comprises administering to a CMV-infected patient a composition

capable of sequestering cmvIL-10 produced by the CMV in the infected patient. The sequestering of the cmvIL-10 is expected to interfere with the CMV life cycle, thereby allowing the infection to be reduced or eliminated.

In yet another aspect of the invention, methods are provided for screening patients for the presence of CMV infection by detecting the presence of cmvIL-10 in serum or tissue samples. Methods suitable for detection of cmvIL-10 in such samples include, but are not limited to, ELISA, Western blotting, radioimmunoassays (RIA), immunoprecipitation, and immunohistochemistry employing antibodies specific for cmvIL-10. Such assays may also be used advantageously on the serum and tissues of organ donor candidates to assess potential donors for the presence of CMV, and on banked blood or plasma supplies to screen for the presence of CMV.

Methods for detecting cmvIL-10-encoding nucleic acids in samples from patients are also featured. The availability of nucleic acid sequence information, such as that provided in SEQ ID NO:1, facilitates the production of specific nucleic acid probes and primers for the detection of cmvIL-10-related nucleic acids in serum or tissue samples. DNA and/or RNA hybridizations may be utilized in such methods. Alternatively, target cmvIL-10 sequences may be increased in a sample by polymerase chain reaction (PCR) amplification. Such amplification facilitates detection of cmvIL-10 encoding nucleic acid sequences. Two pairs of nested primers suitable for use in PCR to amplify the cmvIL-10 gene from the CMV genome present in infected cells are as follows:

1) 5'-TCCTACAGAAACTATTCTAACCGCG-3'(SEQ ID NO:3);
5'-TCATCTTTCCAGCCCGCCTAGCAAC-3' (SEQ ID NO:4); and

2) 5'-CATCATAACATAAAGGACCACCTAC-3'(SEQ ID NO:5);
5'-CGACGCAACGTGGTTAAACAGTACG-3' (SEQ ID NO:6).

The following nested primers may be utilized in reverse-

transcriptase PCR to amplify the cmvIL-10 mRNA/cDNA expressed by CMV infected cells:

1) 5'-GGGACGGCATGCTGCGGCGATGCTG-3' (SEQ ID NO:7); 5'-AGTAACTGGGTGAACGACACCGGAG-3' (SEQ ID NO:8); and

2) 5'-AGGCGCTTCCGAGGAGGCGAAGCCG-3' (SEQ ID NO:9); 5'-GACTGCAAATCGCAACGCTACTTTC-3' (SEQ ID NO:10).

Also featured in the invention are kits useful for detecting or purifying a cmvIL-10 protein or a *cmvIL-10* nucleic acid molecules. The kits typically comprise either antibodies immunologically specific for a cmvIL-10 protein or nucleic acid molecules that specifically hybridize with *cmvIL-10* nucleic acid molecules, along with instructions for using the antibodies or the nucleic acid molecules to detect or purify the cmvIL-10 protein or the *cmvIL-10* nucleic acid molecules. Optionally, they may also comprise one or more reagents for using the antibodies or the nucleic acid molecules to detect or purify the cmvIL-10 protein or the *cmvIL-10* nucleic acid molecules.

Other features and advantages of the present invention will be better understood by reference to the drawings, detailed descriptions and examples that follow.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Region of the CMV genome encoding a protein homologous to IL-10.

Figure 1A: Two sequences encoded by the CMV genome (GenBank Accession # X17403) calculated by the TBLASTN program to have homology with the query sequence from human IL-10 (cIL-10). Numbering on the figure is as follows: human IL-10 (top line of each set) amino acid sequence numbers are used, starting from Met1, segments (portions of SEQ ID NO: 19) are from aa 1-52 and 75-112; CMV genome (bottom line of each set) nucleotide numbers

(portions of SEQ ID NO: 1) are used, based on Genbank Accession No. X17403. The middle line in each set is a consensus with upper case letters indicating amino acid identity, "+" signs indicate amino acid similarity.

Figure 1B: Hydropathy plot of the deduced amino acid sequence from a portion of the CMV genome containing the open reading frame (SEQ ID NO: 18) with homology to the N-terminal portion of human IL-10.

Figure 1C: Region of the CMV genome (SEQ ID NO: 1) encoding the cmvIL-10 protein homologous to cIL-10. Sequences calculated by the TBLAST search program to have homology to cIL-10 are in bold. Nucleotides of the two introns are shown in lower case. Nucleotides of the three exons are shown in upper case. Exon-encoded protein sequences are boxed and shaded. The putative signal peptide is boxed without shading. Nucleotide numbers are those of the CMV genome (GenBank Accession # X17403). Amino acid residues are numbered sequentially, starting from first Met residue and counting only exon-encoded amino acid residues (shaded boxes; collectively, boxed portions comprise SEQ ID NO: 2).

Figure 2. cmvIL-10 expression.

Figure 2A. Western blot analysis of immunoprecipitate from COS-1 cell-conditioned media. COS-1 cells were transiently transfected with plasmid, and after three days one ml of the conditioned media was subjected to immunoprecipitation and Western blotting with anti-FLAG antibody. The molecular weight markers are shown on the left. Cells contained, in lanes from left to right, pEF-SPFL (lane 1, 'mock'), pEF-SPFL-CMV1 (lane 2, cmv_1IL-10), pEF-SPFL-cmv_1-spliced (lane 3, cmv_{1sp}IL-10), pEF-SPFL-cmv_2 (lane 4, cmv_2IL-10), or pEF-SPFL-cmvIL-10 (lane 5, cmvIL-10).

Figure 2B. CMV-infected cells express cmvIL-10. PCR (lanes 3 and 4) or RT-PCR (lanes 6 and 7) with

the same sets of primers was performed with DNA or RNA isolated from virus infected (lanes 4 and 7) or uninfected (lanes 3 and 6) cells as described. Plasmids pEF-cmv_3 (lane 2) and pEF-SPFL-cmvIL-10 (lane 5) were used as positive controls. A 1 kb ladder was run in lanes 1 and 10.

Figure 3. Alignment of the amino acid sequence of human cellular IL-10 and its viral homologs. The amino acid sequences shown include those of cellular IL-10 (cIL-10) encoded by the human genome (SEQ ID NO: 19) and viral IL-10s encoded by following viruses: ebvIL-10, Epstein Barr virus (SEQ ID NO: 20) (Hsu et al., (1990) Science 250: 830-832); ovIL-10, Orf poxvirus (SEQ ID NO: 21)(Flemming et al. (1997) J. Virol. 71: 4857-4861); and cmvIL-10, cytomegalovirus (SEQ ID NO: 2). The consensus sequence is shown on the bottom. Amino acid residues identical to those in the corresponding position of the consensus sequence are shown in bold. Amino acid residues similar to those in the corresponding position of the consensus sequence are shown in lower case. The numbering of amino acid residues begins at the first Met of the signal peptide.

α helices A through F from the crystal structure (Zdanov et al., (1995) Structure (London) 3: 591-601; Zdanov et al., (1997) J. Mol. Biol. 268: 460-467), are underlined. Symbols ① and ② are placed above the Cys residues of which form intramolecular disulfide bridges 1 and 2 respectively. The symbol ⊖ points to one additional Cys residue of cmvIL-10. Asterisks (*) denote amino acids residues predicted to be involved in interaction with IL-10R1 (Zdanov et al., (1996) Protein Sci. 5: 1955-1962) Symbol ■ points to amino acids residues conserved within regions which interact with IL-10R1. Symbol □ points to amino acid residues conserved

in a portion of IL-10 homologs which may be involved in interaction with IL-10R2. Arrows indicate positions of introns within cIL-10 and cmvIL-10 genes. Numbers in parenthesis represent the intron number in cIL-10 and cmvIL-10 (intron # within cIL-10/intron # within cmvIL-10).

Figure 4. Ligand binding and biological assays performed on hamster cells.

Figure 4. (Top Rows) Representation of four cell lines used in these experiments: the parental Chinese hamster 16-9 cells and three 16-9-based cell lines expressing either human IL-10R1/γR1 chimeric receptor or human IL-10R2 alone or both receptors together (Kotenko et al., (1997) EMBO J. 16: 5894-5903).

Figure 4. (Third Row) Each of the four cell lines described in Fig 4A were incubated for 30 min. at 4° C with conditioned medium from COS-1 cells transfected with one of the following plasmids: the control, pEF-SPFL (open areas, thick lines); pEF-SPFL-cIL-10 (open areas, thin lines) or pEF-SPFL-cmvIL-10 (shaded areas, thin lines) (panels A, B, C, and D). Ligand binding to the cell surface was determined by flow cytometry with anti-FLAG antibody (Sigma) as the primary antibody and fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Santa Cruz) as the secondary antibody. Here, and in Figure 4, Row Four, the ordinate represents relative cell number; the abscissa values are relative fluorescence.

Figure 4. (Row Four) The ability of cIL-10 and cmvIL-10 to induce MHC class I antigen expression was demonstrated by flow cytometry as described in Kotenko et al., 1997. The cells described in Fig 4A were left untreated (open areas, thick lines) or treated with conditioned media (100 µl) from COS-1 cells transfected with plasmid pEF-SPFL-cmvIL-10 (shaded areas, thin lines) or with cIL-10 (100 units/ml) (open areas, thin lines)

(panels E, F, G, and H).

Figure 5. Ligand binding competition as determined via flow cytometry. Cells expressing both chains of the IL-10 receptor complex were incubated with FL-cmvIL-10 alone (30 µl of conditioned media from COS-1 cells expressing FL-cmvIL-10; open area, thick line) or together with concentrations of cIL-10 (expressed as ng/ml; open areas, thin lines) (panel I); or in panel J with FL-cIL-10 alone (30 µl of conditioned media from COS-1 cells expressing FL-cIL-10; open area, thick line) or together with aliquonts of conditioned media from COS-1 cells expressing cmvIL-10 (expressed as µl; open areas, thin lines).

Figure 6. cmvIL-10-induced Stat activation in hamster cells and PBMCs. Electrophoretic mobility-shift assay (EMSA) was used to demonstrate that cmvIL-10 activates the Stat1 and Stat3 DNA-binding complexes. Hamster cells expressing both receptor chains (as described in Fig. 4 (Top Rows)) and PBMCs were used. Cells were left untreated or treated with recombinant IL-10 (100 units/ml) or with conditioned media (200 µl) from COS cells transfected with the pEF-SPFL-cmvIL-10 plasmid or from uninfected cells or CMV-infected cells. Cellular lysates were prepared and assayed for Stat activation in the EMSA as described previously (Kotenko et al., 1997, supra). Positions of Stat DNA-binding complexes are indicated by arrows. Antibodies against Stat1 and Stat3 were added as indicated to reduce the mobility of complexes containing these proteins.

Figure 7. Schematic map of the CMV genome. The CMV genome is organized as two regions of unique sequences, unique long (U_L) and unique short (U_S), flanked by two sets of inverted repeats (TR_L/IR_L) and (IR_S/TR_S) (light shaded boxes). *Ban*II and *Xho*I are sites for digestion with restriction endonucleases within mtrII

region. The 79 ORF (dark shaded box) is an open reading frame of 79 amino acids whose disruption abolishes mtrII transforming ability. cmvIL-10 is encoded by three exons (exon-encoded amino acid sequences are represented by medium-dark shaded boxes, spliced regions are represented by open boxes with dotted lines). SP is a signal peptide of the cmvIL-10 (light shaded box).

DETAILED DESCRIPTION OF THE INVENTION

I. Definitions

Various terms relating to the biological molecules of the present invention are used hereinabove and also throughout the specifications and claims.

With reference to nucleic acid molecules, the term "isolated nucleic acid" is sometimes used. This term, when applied to DNA, refers to a DNA molecule that is separated from sequences with which it is immediately contiguous (in the 5' and 3' directions) in the naturally occurring genome of the organism from which it was derived. For example, the "isolated nucleic acid" may comprise a DNA molecule inserted into a vector, such as a plasmid or virus vector, or integrated into the genomic DNA of a procaryote or eucaryote. An "isolated nucleic acid molecule" may also comprise a cDNA molecule.

With respect to RNA molecules, the term "isolated nucleic acid" primarily refers to an RNA molecule encoded by an isolated DNA molecule as defined above. Alternatively, the term may refer to an RNA molecule that has been sufficiently separated from RNA molecules with which it would be associated in its natural state (i.e., in cells or tissues), such that it exists in a "substantially pure" form (the term "substantially pure" is defined below).

With respect to proteins or peptides, the term "isolated protein (or peptide)" or "isolated and purified

protein (or peptide)" is sometimes used herein. This term refers primarily to a protein produced by expression of an isolated nucleic acid molecule of the invention. Alternatively, this term may refer to a protein which has been sufficiently separated from other proteins with which it would naturally be associated, so as to exist in "substantially pure" form.

The term "substantially pure" refers to a preparation comprising at least 50-60% by weight the compound of interest (e.g., nucleic acid, oligonucleotide, protein, etc.), absent water, salts and common buffer components. More preferably, the preparation comprises at least 75% by weight, and most preferably 90-99% by weight, the compound of interest. Purity is measured by methods appropriate for the compound of interest (e.g. chromatographic methods, agarose or polyacrylamide gel electrophoresis, HPLC analysis, and the like).

Nucleic acid sequences and amino acid sequences can be compared using computer programs that align the similar sequences of the nucleic or amino acids thus define the differences. For instance, the BLAST programs used to query sequence similarity in GenBank and other public databases may be used. The GCG Wisconsin Package version 9.1, available from the Genetics Computer Group in Madison, Wisconsin, and the default parameters used (gap creation penalty=12, gap extension penalty=4) by that program may also be used to compare sequence identity and similarity.

The term "substantially the same" refers to nucleic acid or amino acid sequences having sequence variation that do not materially affect the nature of the protein (i.e. the structure, stability characteristics, substrate specificity and/or biological activity of the protein). With particular reference to nucleic acid

sequences, the term "substantially the same" is intended to refer to the coding region and to conserved sequences governing expression, and refers primarily to degenerate codons encoding the same amino acid, or alternate codons encoding conservative substitute amino acids in the encoded polypeptide. With reference to amino acid sequences, the term "substantially the same" refers generally to conservative substitutions and/or variations in regions of the polypeptide not involved in determination of structure or function.

The terms "percent identical" and "percent similar" are also used herein in comparisons among amino acid and nucleic acid sequences. When referring to amino acid sequences, "percent identical" refers to the percent of the amino acids of the subject amino acid sequence that have been matched to identical amino acids in the compared amino acid sequence by a sequence analysis program. "Percent similar" refers to the percent of the amino acids of the subject amino acid sequence that have been matched to identical or conserved amino acids. Conserved amino acids are those which differ in structure but are similar in physical properties such that the exchange of one for another would not appreciably change the tertiary structure of the resulting protein. Conservative substitutions are defined in Taylor (1986, J. Theor. Biol. 119:205). When referring to nucleic acid molecules, "percent identical" refers to the percent of the nucleotides of the subject nucleic acid sequence that have been matched to identical nucleotides by a sequence analysis program.

With respect to antibodies, the term "immunologically specific" refers to antibodies that bind to one or more epitopes of a protein of interest, but which do not substantially recognize and bind other molecules in a sample containing a mixed population of

antigenic biological molecules.

With respect to oligonucleotides or other single-stranded nucleic acid molecules, the term "specifically hybridizing" refers to the association between two single-stranded nucleic acid molecules of sufficiently complementary sequence to permit such hybridization under pre-determined conditions generally used in the art (sometimes termed "substantially complementary"). In particular, the term refers to hybridization of an oligonucleotide with a substantially complementary sequence contained within a single-stranded DNA or RNA molecule, to the substantial exclusion of hybridization of the oligonucleotide with single-stranded nucleic acids of non-complementary sequence.

A "coding sequence" or "coding region" refers to a nucleic acid molecule having sequence information necessary to produce a gene product, when the sequence is expressed.

The term "operably linked" or "operably inserted" means that the regulatory sequences necessary for expression of the coding sequence are placed in a nucleic acid molecule in the appropriate positions relative to the coding sequence so as to enable expression of the coding sequence. This same definition is sometimes applied to the arrangement other transcription control elements (e.g. enhancers) in an expression vector.

Transcriptional and translational control sequences are DNA regulatory sequences, such as promoters, enhancers, polyadenylation signals, terminators, and the like, that provide for the expression of a coding sequence in a host cell.

The terms "promoter", "promoter region" or "promoter sequence" refer generally to transcriptional regulatory regions of a gene, which may be found at the

5' or 3' side of the coding region, or within the coding region, or within introns. Typically, a promoter is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. The typical 5' promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence is a transcription initiation site (conveniently defined by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

A "vector" is a replicon, such as plasmid, phage, cosmid, or virus to which another nucleic acid segment may be operably inserted so as to bring about the replication or expression of the segment.

The term "nucleic acid construct" or "DNA construct" is sometimes used to refer to a coding sequence or sequences operably linked to appropriate regulatory sequences and inserted into a vector for transforming a cell. This term may be used interchangeably with the term "transforming DNA". Such a nucleic acid construct may contain a coding sequence for a gene product of interest, along with a selectable marker gene and/or a reporter gene.

The term "selectable marker gene" refers to a gene encoding a product that, when expressed, confers a selectable phenotype such as antibiotic resistance on a transformed cell.

The term "reporter gene" refers to a gene that encodes a product which is easily detectable by standard methods, either directly or indirectly.

A "heterologous" region of a nucleic acid

construct is an identifiable segment (or segments) of the nucleic acid molecule within a larger molecule that is not found in association with the larger molecule in nature. Thus, when the heterologous region encodes a mammalian gene, the gene will usually be flanked by DNA that does not flank the mammalian genomic DNA in the genome of the source organism. In another example, coding sequence is a construct where the coding sequence itself is not found in nature (e.g., a cDNA where the genomic coding sequence contains introns, or synthetic sequences having codons different than the native gene). Allelic variations or naturally-occurring mutational events do not give rise to a heterologous region of DNA as defined herein.

A cell has been "transformed" or "transfected" by exogenous or heterologous DNA when such DNA has been introduced inside the cell. The transforming DNA may or may not be integrated (covalently linked) into the genome of the cell. In prokaryotes, yeast, and mammalian cells for example, the transforming DNA may be maintained on an episomal element such as a plasmid. With respect to eukaryotic cells, a stably transformed cell is one in which the transforming DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the transforming DNA. A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth *in vitro* for many generations. The media in which cells have been grown under the conditions specified is referred to as "conditioned media" or sometimes as "spent media".

II. Description

Provided in accordance with the present invention is a new viral interleukin 10, homologous to cellular IL-10, and encoded by the UL111a open reading frame of the CMV genome. Though the existence of this open reading frame has been known for years, its function heretofore had not been elucidated.

Although the homology between cIL-10 and cmvIL-10 is limited (27% a.a. identity), cmvIL-10 binds to and induces signal transduction through the same IL-10 receptor complex as cIL-10. However, cmvIL-10 exhibits the ability to cause cellular transformation.

Results obtained in accordance with the present invention demonstrate that a 79-amino-acid open reading frame within the morphological transforming region II (mtrII) in the CMV genome, which was previously identified as responsible for malignant transformation (Razzaque et al., (1988) Proc. Natl. Acad. Sci. USA: 85: 5706-5713), is colinear with cmvIL-10. Results obtained in accordance in the present work further showed that NIH3T3 cells expressing cmvIL-10 were able to form tumors in syngeneic Swiss mice, therefore, it is clear that CMV can support tumor growth and cell transformation, through the production of cmvIL-10. CMV infection has been associated with a variety of human cancers; the production of cmvIL-10 may be the major mechanism by which CMV supports malignant transformation.

The cmvIL-10 of the present invention has great practical utility, first as a substitute for cIL-10 in various therapeutic uses; and second, as a target for control or eradication of CMV-associated disease.

Now that a function of the UL111a open reading frame has been discovered, the present invention encompasses a useful embodiment of that discovery, which

is the UL111a open reading frame isolated and inserted into a heterologous vector suitable for expressing the IL-10 homolog, cmvIL-10. Preferably, the vector comprises the segment of the UL111a ORF that maps between 159675 and 160376 of the CMV genome (Fig 1C, SEQ ID NO:1). Expression of this sequence produces a 175 amino acid polypeptide which is the cmvIL-10 (Fig. 3, SEQ ID NO:2).

This invention is intended to encompass nucleic acid molecules and their encoded proteins that are natural variants or mutants of SEQ ID NOS: 1 or 2, which are likely to be found in different isolates of CMV. Because such variants are expected to possess certain differences in nucleotide and/or amino acid sequence, this invention provides an isolated cmvIL-10-encoding nucleic acid molecule having at least about 60%, preferably 70%, more preferably 80%, and even more preferably over 90% sequence homology across SEQ ID NO:1; and, most preferably, with such homology present specifically across the region comprising the coding sequence of SEQ ID NO:1.

This invention also provides isolated polypeptide products of the open reading frames of SEQ ID NO:1, having at least about 60%, preferably 70%, 80%, 90% or greater, sequence homology with the amino acid sequences of SEQ ID NO:2. Because of the natural sequence variation likely to exist among CMV isolates, one skilled in the art might expect to find at least about 30-40% nucleotide sequence variation, while still maintaining the unique properties of the cmvIL-10 protein of the present invention. For example, several CMV isolates have been examined and at least two variations have been identified in different CMV laboratory strains. These are as follows: 1) an amino acid Thr has been inserted after Ala26 and; 2) a substitution of Ile94 by

Thr94 (numbering as in Figs. 1C and 3) has also been observed. The expectation of minor sequence variations is due in part to the degeneracy of the genetic code, as well as to the known evolutionary success of conservative amino acid sequence variations, which do not appreciably alter the nature of the encoded protein. Accordingly, such variants are considered substantially the same as one another, and are included within the scope of the present invention.

The cmvIL-10-encoding nucleic acid molecules of the invention may be prepared by two general methods: (1) they may be synthesized from appropriate nucleotide triphosphates or their analogs, or (2) they may be isolated from biological sources. Both methods utilize protocols well known in the art.

The availability of nucleotide and amino acid sequence information, such as SEQ ID NO:1 and SEQ ID NO:2, enables preparation of an isolated nucleic acid molecule of the invention by oligonucleotide synthesis. Synthetic oligonucleotides may be prepared by the phosphoramadite method employed in the Applied Biosystems 38A DNA Synthesizer or similar devices. The resultant construct may be purified according to methods known in the art, such as high performance liquid chromatography (HPLC).

Variants of SEQ ID NO:1 also may be synthesized as described above. For instance, in some cases it may be advantageous to customize a nucleic acid molecule encoding SEQ ID NO:2, or functional equivalent thereof, for expression in cells of a particular species. In this case, SEQ ID NO: 2 may be back-translated to generate a sequence with appropriate codon usage preferences for the selected species, as well as any other features known to enhance gene expression in that species. Codon usage preference tables for a wide variety of species are

published, and computer programs for performing reverse translations are available. In a particularly preferred embodiment, the back-translated nucleic acid molecule encodes SEQ ID NO:2. In another preferred embodiment, it encodes a variant of SEQ ID NO:2 wherein selected residues of the polypeptide comprise conservative substitutions for the corresponding residue found in SEQ ID NO:2. In yet another preferred embodiment, the nucleotide sequence contains one or more specific single nucleotide polymorphisms (SNPs) which provide properties of interest, such as altered binding, altered biological activity or altered expression, altered specificity or altered therapeutic spectrum of either the nucleotide sequence or the encoded cmvIL-10 protein. Such SNPs are contemplated and considered to be within the scope of the present invention.

The cmvIL-10-encoding nucleic acids also may be prepared from different isolates of CMV, using methods known in the art. In accordance with the present invention, nucleic acids having the appropriate level sequence homology with a part or all of the coding regions of SEQ ID NO:1 may be identified by using hybridization and washing conditions of appropriate stringency. For example, hybridizations may be performed, according to the method of Sambrook et al. (1989), using hybridization solution comprising: 5X SSC, 5X Denhardt's reagent, 1.0% SDS, 100 µg/ml denatured, fragmented salmon sperm DNA, 0.05% sodium pyrophosphate, and up to 50% formamide. Hybridization is carried out at 37-42 °C for at least 6 h. Following hybridization, filters are washed as follows: (1) 5 min at room temperature in 2X SSC and 1% SDS; (2) 15 min at room temperature in 2X SSC and 0.1% SDS; (3) 30 min - 1 h at 37 °C in 2X SSC and 0.1% SDS; (4) 2 h at 45-55 °C in 2X SSC and 0.1% SDS, changing the solution every 30 min.

One common formula for calculating the stringency conditions required to achieve hybridization between nucleic acid molecules of a specified sequence homology (Sambrook et al., 1989):

$T_m = 81.5\ °C + 16.6 \text{Log}\ [Na+] + 0.41(\%\ G+C) - 0.63\ (\%\ \text{formamide}) - 600/\#\text{bp in duplex}$

As an illustration of the above formula, using [N+] = [0.368] and 50% formamide, with GC content of 42% and an average probe size of 200 bases, the T_m is 57 °C. The T_m of a DNA duplex decreases by 1 - 1.5 °C with every 1% decrease in homology. Thus, targets with greater than about 75% sequence identity would be observed using a hybridization temperature of 42 °C. Such a sequence would be considered substantially homologous to the sequences of the present invention. In a preferred embodiment, the hybridization is at 37 °C and the final wash is at 42 °C, in a more preferred embodiment the hybridization is at 42 °C and the final wash is at 50 °C, and in a most preferred embodiment the hybridization is at 42 °C and final wash is at 65 °C, with the above hybridization and wash solutions. Conditions of high stringency include hybridization at 42 °C in the above hybridization solution and a final wash at 65 °C in 0.1X SSC and 0.1% SDS for 10 min.

Nucleic acids of the present invention may be maintained as DNA in any convenient cloning vector. In a preferred embodiment, clones are maintained in plasmid cloning/expression vectors. For example, for expression in eucaryotic cells pcDEF3, pcDNA3, pTet-Tta and their derivatives may be utilized. In insect cells, pFastBac and its derivatives are suitable for expression of the cmvIL-10 molecules of the invention. For expression in procaryotic cells pTGATG, pGEX (GST-fusion system), pMAL (MBP-fusion system), pQE (6xHis-fusion system) and their

derivatives may be employed. The use of retroviral vectors encoding cmvIL-10 are also contemplated to be within the scope of the present invention.

The cmvIL-10-encoding nucleic acid molecules of the invention include genomic or recombinant DNA, RNA, and fragments thereof; any of which may be single- or double-stranded. Thus, this invention provides oligonucleotides (sense or antisense strands of DNA or RNA) having sequences capable of hybridizing with at least one sequence of a nucleic acid molecule of the present invention, such as selected segments of the DNA having SEQ ID NO:1. Such oligonucleotides are useful as probes for detecting cmvIL-10-encoding genes or mRNA in test samples, e.g. by PCR amplification, or for the positive or negative regulation of expression of cmvIL-10 genes at or before translation of the mRNA into proteins.

The cmvIL-10 protein may be prepared in a variety of ways, according to known methods. If produced *in situ* the polypeptides may be purified from appropriate sources, e.g., virus preparations.

Alternatively, the availability of nucleic acid molecules encoding the polypeptides enables production of the proteins using *in vitro* expression methods known in the art. For example, a gene may be cloned into an appropriate *in vitro* transcription vector, such as pSP64 or pSP65 for *in vitro* transcription, followed by cell-free translation in a suitable cell-free translation system, such as wheat germ or rabbit reticulocytes. Complete kits of *in vitro* transcription/translation systems are commercially available, e.g., from Promega Biotech, Madison, Wisconsin or BRL, Rockville, Maryland.

According to a preferred embodiment, larger quantities of cmvIL-10 may be produced by expression in a suitable procaryotic or eucaryotic system. For example, part or all of a DNA molecule, such as the coding portion

of SEQ ID NO:1, may be inserted into a plasmid vector adapted for expression in a bacterial cell (such as *E. coli*) or a yeast cell (such as *Saccharomyces cerevisiae*), or into a baculovirus vector for expression in an insect cell. Such vectors comprise the regulatory elements necessary for expression of the DNA in the host cells, positioned in such a manner as to permit expression of the DNA in the host cells. Such regulatory elements required for expression include, but are not limited to promoter sequences, transcription initiation sequences and, optionally, enhancer sequences.

The cmvIL-10 protein produced by expression in a recombinant procaryotic or eucaryotic system may be purified according to methods known in the art. In a preferred embodiment, a commercially available expression/secretion system can be used, whereby the recombinant protein is expressed and thereafter secreted from the host cell, to be easily purified from the surrounding medium. An alternative approach to expression/secretion vectors requires purifying the recombinant protein by affinity separation, such as by immunological interaction with antibodies that bind specifically to the recombinant protein. Such methods are commonly used by skilled practitioners. A preferred embodiment comprises expression of a his-tagged cmvIL-10 protein, followed by separation from cellular debris and spent media via affinity methods, such as immobilized metal affinity chromatography (IMAC).

The present invention also provides antibodies capable of immunospecifically binding to polypeptides of the invention. Polyclonal or monoclonal antibodies directed toward cmvIL-10 may be prepared according to standard methods. Monoclonal antibodies may be prepared according to general methods of Köhler and Milstein, following standard protocols. In a preferred embodiment,

antibodies immunospecifically recognize discrete epitopes of cmvIL-10 such as those comprising the active site(s) or receptor binding region(s) of the protein.

The CMV-encoded IL-10 homolog of the present invention will find therapeutic utility in any situation where cellular IL-10 is presently employed. As described above, cellular IL-10 is a powerful anti-inflammatory agent and a potent immunosuppressor. Experimental results, obtained in accordance with the present invention, indicate that cmvIL-10 binds with higher affinity to the IL-10 receptor complex than the cellular IL-10. As a result, cmvIL-10 is likely to be a more potent inducer of biological activities than the cellular IL-10 presently in use.

Cellular IL-10 is currently being assessed in clinical trials for the treatment of various pathological conditions, including Crohn's disease, septic shock, and psoriasis. Also IL-10 or IL-10-neutralizing agents have potential therapeutic value in the treatment of rheumatoid arthritis, Systemic Lupus Erythematosus, multiple sclerosis (MS), as well as certain cancers. Thus, according to another aspect of the invention, pharmaceutical formulations are provided for the treatment of pathological conditions that respond to IL-10 therapy. These formulations comprise an effective therapeutic amount of the cmvIL-10 of the invention, or an isolated nucleic acid encoding cmvIL-10, in a suitable pharmaceutical medium or carrier.

The discovery of this novel IL-10 homolog, cmvIL-10, also has significant implications relating to the treatment of CMV-associated diseases, including (1) CMV-related cancers, (2) acute CMV infection in immunocompromised individuals, such as transplant recipients and AIDS patients, (3) congenital birth defects associated with CMV and (4) atherosclerosis,

restenosis after coronary angioplasty, chronic rejection in organ transplant and chronic graft-versus-host disease following bone marrow transplant. In treatment of such diseases and conditions, biological molecules that can bind and sequester the cmvIL-10 produced by CMV could interrupt the CMV life cycle, thereby reducing or eliminating the CMV-related disease or condition.

Biological molecules capable of binding and sequestering cmvIL-10 include, but are not limited to, any combination of one or more of (1) soluble IL-10 receptors, (2) immunoadhesion molecules comprising the extracellular domain of the IL-10 receptor fused to Fc fragments of IgG, and (3) anti-cmvIL-10 antibodies.

The discovery of a cmvIL-10 in accordance with the invention also enables a new means for detecting the presence or quantity of CMV in a test sample from a patient. Such detection methods comprise standard immunological detection of the protein, or standard hybridization-based detection of the cmvIL-10-encoding mRNA, in the test sample.

Detection and/or purification of cmvIL-10 or its encoding nucleic acid is facilitated by packaging appropriate reagents into kits, which are another feature of the present invention. Such kits may comprise antibodies or hybridizing probes, instructions for use of the kits, and optional reagents for carrying out detection or purification methods.

The following example is provided to describe the invention in greater detail; it is intended to illustrate, not to limit, the invention.

EXAMPLE 1
Identification of a Cytomegalovirus-Encoded IL-10 Homolog Capable of Cellular Transformation

Searches of the GenBank database with the TBLASTN program for possible IL-10 homologs revealed, in close proximity, two regions of the CMV genome with partial homology to the IL-10 sequence (Fig. 1A). The reading frame corresponding to the first region of homology had an ATG codon just upstream of the region where the calculated homology begins (Fig. 1A). A hydropathy plot of this region indicated that the amino terminus of the predicted protein is highly hydrophobic (Fig. 1B), suggesting that the sequence could encode a signal peptide.

To determine whether the two separate regions of homology, in different reading frames, represent two exons of one gene and can be spliced together, the size of the protein encoded by this region of the CMV genome was examined.

A PCR-derived fragment, designated cmv_1 (spanning nucleotides 159735-160178 of the CMV genome, Fig. 1C), that encompassed the first region of calculated homology starting at the nucleotide sequence corresponding to Ser20, the first amino acid after the hypothetical signal peptide; along with the entire second region of calculated homology, in a separate reading frame, was cloned into plasmid pEF-SPFL.

Plasmid pEF-SPFL is a derivative of the pcDEF3 vector (Goldman et al., (1996) BioTechniques 21: 1013-1015) wherein the fragment encoding the human IFN-γR2 signal peptide, followed by the FLAG epitope (Soh et al., (1994) Cell 76: 793-802; Kotenko et al., (1999) Proc. Natl. Acad. Sci. USA 96: 5007-5012) is introduced into *Kpn*I and *Bam*HI sites of the pcDEF3 vector. Primers 5'-TAGGATCCTTCCGAGGAGGCGAAG-3' (SEQ ID NO: 11) and 5'-

ATGAATTCGTTGTTACCTCT-3' (SEQ ID NO: 12) and C101AD135-175 cosmid DNA were used for PCR cloning of the cmv_1 fragment into plasmid pEF-SPFL with the use of *Bam*HI and *Eco*RI restriction endonucleases.

The resultant plasmid, pEF-SPFL-cmv_1, contained cloned within it, the first region of calculated homology to IL-10 adjacent to and in frame with the FLAG epitope.

COS-1 cells were transiently transfected with plasmid pEF-SPFL-cmv_1. Three days after transfection, conditioned media was collected and subjected to Western blot analysis, the results of which are shown in Figure 2. The molecular weight of the protein potentially encoded by the portion of the reading frame corresponding to the first region of calculated homology was approximately 8 kDa; however, a protein of about 14 kDa, immunoreactive with anti-FLAG antibody, was detected. (Fig. 2, second lane). In order to explain this result, the mRNA transcript corresponding to the 14 kDa protein produced was cloned via reverse transcriptase and PCR (RT-PCR) from COS-1 cells transfected with plasmid pEF-SPFL-cmv_1.

Primers of the same sequence as those used for the creation of pEF-SPFL-cmv_1 were used, with total RNA isolated from COS-1 cells transfected with plasmid pEF-SPFL-cmv_1, for RT-PCR to ultimately clone the putative cmv_1-spliced fragment into plasmid pEF-SPFL with the use of *Bam*HI and *Eco*RI restriction endonucleases, resulting in the plasmid referred to as pEF-SPFL-$cmv_{1\text{-spliced}}$.

Analysis showed that in the cloned cmv_1 RT-PCR-derived ($cmv_{1\text{-spliced}}$) fragment, the region spanning nucleotides 159858 to 159933 had been spliced out (Fig. 1C), indicating that in this region of the CMV genome there is a small intron which had been excised. The splicing event connected the two frames containing the IL-10 homology; however, the COS-1 cell conditioned

medium still failed to induce Stat activation in hamster cells with the reconstituted human IL-10 receptor complex (Kotenko et al., 1997, supra) or in human peripheral blood mononuclear cells (PBMCs). In addition, when the predicted amino acid sequence encoded by the $cmv_{1\text{-spliced}}$ fragment was aligned with the IL-10 sequence, the portion corresponding to the C-terminal third of the IL-10 protein was not represented in the $cmv_{1\text{-spliced}}$-encoded sequence. The hypothesis was that a third portion of the CMV genome encoded the missing C-terminal part of the CMV-encoded IL-10-like protein.

A longer fragment of the CMV genome (herein designated cmv_2), mapped between nucleotides 159735-160376 (Fig. 1C), was therefor cloned into plasmid pEF-SPFL via PCR. Primers 5'-TAGGATCCTTCCGAGGAGGCGAAG-3' (SEQ ID NO: 11) and 5'-AGCGGAATTCAAATCGCAACGC-3' (SEQ ID NO: 13) and C101AD135-175 cosmid DNA were used for the PCR cloning of the cmv_2 fragment into plasmid pEF-SPFL vector, with the use of *Bam*HI and *Eco*RI restriction endonucleases, resulting in plasmid pEF-SPFL-cmv_2.

The Western blot (Fig. 2) showed the presence of an anti-FLAG antibody-recognized protein of approximately 21 kDa where previously only a 14 kDa protein had been detected. A protein of 21kDa is of comparable mass to that of cellular IL-10. The cmv_2-derived mRNA corresponding to this 21kDa protein was cloned from transfected COS-1 cells via RT-PCR, and sequenced.

Primers of the same sequence as those used for the cloning of plasmid pEF-SPFL-cmv_2 and total RNA isolated from COS-1 cells transfected with plasmid pEF-SPFL-cmv_2 were used for the RT-PCR cloning of the cmv_2-derived fragment ultimately into plasmid pEF-SPFL with the use of *Bam*HI and *Eco*RI restriction endonucleases, resulting in plasmid pEF-SPFL-cmvIL-10.

In addition to the presence of a first intron determined in previous experiments, the RT-PCR fragment was missing the sequence spanning nucleotides 160135-160217 of the CMV genome, indicating the presence of a second intron within this region of the CMV genome (Fig. 1C). As a result of splicing, a frame shift occurred to bring the complete protein-encoding sequence into a common reading frame (Fig. 1C). The resulting open reading frame encoded a protein of 175 amino acids (Fig. 1C and Fig. 3) which was designated cmvIL-10. Medium from COS-1 cells transfected with the expression vector pEF-SPFL-cmvIL-10 was analyzed by Western blotting, using an anti-FLAG antibody, and revealed a 21 kDa band which comigrated with cmv_2IL-10 (Fig 2A. Lane 5, FL-cmv-IL-10; cf. Lane 4, FL-cmv_2IL-10). After longer exposure, several additional bands were observed in the region of approximately 30 - 35 kDa, suggesting possible glycosylation of cmv-IL-10. Indeed, there is a site for N-linked glycosylation, Asn-151-X-Thr-153. Treatment of the conditioned medium with Peptide: *N*-glycosidase F resulted in the disappearance of the higher bands and enhancement of the 21 kDa band, consistent with glycosylation of the 30 - 35 kDa proteins.

The complete sequence of the CMV strain AD169 genome was determined in 1990, but the functions of many of the ORFs within the CMV genome have yet to be discovered. The present study identifies the function of the UL111a ORF. The gene encodes a viral homolog of cellular IL-10, so that it was designated cmvIL-10, thus extending the number of herpes viruses harboring homologs of IL-10. The cmvIL-10 reveals a number of distinct features when compared with other viral IL-10 homologs encoded by herpesviruses that are able to infect humans, including EBV and OV. Alignment of the predicted CMV-encoded IL-10 (cmvIL-10) with human IL-10 and other

viral-encoded homologs which are active on human cells (Fig. 3) revealed only 27 % amino acid identity to the human IL-10 gene sequence, whereas other vIL-10s are approximately 85% identical with human IL-10.

The cmvIL-10 gene has a unique structure. The cIL-10 gene is composed of 5 exons (GenBank Accession # U16720), the position of the two introns within the cmvIL-10 gene corresponds to the position of the first and third introns of the cIL-10 gene (Figures 1 and 3), suggesting the possibility that these genes diverged from a common ancestral gene. The 5' and 3' intron/exon splice sites (Figure 1C) for both introns within cmvIL-10 gene conform well to consensus sequences (exon/GT - intron- AG/exon). In contrast, ovIL-10 and ebvIL-10 genes do not have introns. This suggests that CMV might have captured a partially-spliced IL-10 mRNA sequence from infected cells or alternatively, CMV might have captured the human IL-10 gene which subsequently evolved to eliminate two introns and shorten the remaining two.

The active IL-10 receptor complex is composed of two subunits, the ligand binding chain, IL-10R1 and the second chain, IL-10R2, which is required for signaling (Kotenko et al., 1997, supra). To determine whether cmvIL-10 can bind and signal through the human IL-10 receptor complex, four hamster cell lines in which the native receptor complex was modified to facilitate the detection of the IL-10-induced biological activities (Kotenko et al., 1997, supra) were used, each expressing different components of the modified human IL-10 receptor complex (Fig. 4, top 2 rows). In these lines, the IL-10R1 intracellular domain was substituted by the IFN-γR1 intracellular domain. With this substitution, IL-10 can activate IFN-γ-like biological responses, such as MHC class I antigen induction and Stat1 activation, in those cells expressing the chimeric IL-10R1/γR1 chain together

with the intact second chain, IL-10R2 (Kotenko et al., 1997, supra).

Parental hamster CHO-derived 16-9 cells and each of three cell lines expressing either the receptor subunits individually or together were used in ligand binding and cellular activation experiments (Fig. 4).

To create cIL-10 protein tagged with FLAG epitope at the N-terminus, the primers 5'-CGGGATCCCAGCC CAGGGCAGGGCACC-3' (SEQ ID NO: 14), and 5'-GCTCTAGATCAGTTTCGTATCTTCAT-3' (SEQ ID NO: 15), and cIL-10-encoded plasmid DNA (Viera et al., (1991) Proc. Natl. Acad. Sci. USA 88: 1172-1176) were used for PCR-based cloning. The PCR fragment was cloned into the pEF-SPFL vector with the use of *Bam*HI and *Xba*I restriction endonucleases. COS-1 cells were transfected with resultant plasmid and the expressed FLAG epitope-tagged cIL-10 (FL-cIL-10) was purified on an anti-FLAG affinity column. FLAG epitope-tagged cmvIL-10 (FL-cmvIL-10) was made in the analogous manner. FLAG epitope-tagged cmvIL-10 (FL-cmvIL-10) and cIL-10 (FL-cIL-10) were used to detect ligand binding by flow cytometry (Fig. 4, third row).

The experiments demonstrated that cmvIL-10 binds to the cell surface of hamster cells expressing IL-10R1/γR1 alone or with IL-10R2, but not to the parental 16-9 cells or cells expressing IL-10R2 alone (Fig. 4, third row, panels A-D). Furthermore, cIL-10 competes for receptor binding with FL-cmvIL-10 in a concentration-dependent manner on cells expressing both chains of the IL-10 receptor complex (Fig. 5, panel A). Conversely, cmvIL-10 can also compete in a concentration-dependent manner with FL-cIL-10 (Fig. 5, panel B).

It was determined that cmvIL-10 signals through the human IL-10 receptor complex. Since the chimeric IL-10 receptor complex with the intracellular domain of the

IL-10R1 replaced by the IFN-γR1 intracellular domain was used, IL-10 activated IFN-γ-like responses. As previously demonstrated with cellular IL-10 (Kotenko et al., 1997, supra), only hamster cells expressing both subunits of the modified IL-10 receptor complex were capable of cmvIL-10 induced MHC class I antigen expression (Fig. 4, row four, panels E-H) and Stat1 activation, as measured by electrophoretic mobility-shift assay (EMSA)(Fig. 6).

PBMCs were used to demonstrate that cmvIL-10 activated the same pattern of the Stat1 and Stat3 DNA-binding complexes characteristic of cIL-10 signaling (Fig. 6).

To demonstrate that cmvIL-10 can be secreted via its own signal peptide and can bind and activate the IL-10 receptor complex, the PCR-derived fragment of the CMV genome between nucleotides 159670 to 160376 (Fig. 1C) was cloned into the pcDEF3 vector (Goldman et al. 1996, supra). To facilitate the cloning, primers 5'-CGGGATCCTGCGGCGATGCTG-3' (SEQ ID NO: 16) and 5'-AGCGGAATTCAAATCGCAACGC-3' (SEQ ID NO: 17) and C101AD135-175 cosmid DNA were used to enable the PCR cloning of the cmvIL-10-encoding sequences including the sequence encoding the putative signal peptide. The resultant PCR fragment was cloned into the pcDEF3 vector with the use of *Bam*HI and *Eco*RI restriction endonucleases, resulting in the plasmid designated herein as pEF-cmv_3.

The conditioned medium from COS-1 cells transiently transfected with this plasmid was used for competitive binding with FL-cIL-10, as well as in MHC class I induction experiments and in the EMSA experiments. cmvIL-10 produced with its own putative signal peptide was able to compete with FL-cIL-10 binding as shown earlier (Fig 4, panel J) and demonstrated the same activities as FL-cmvIL-10 (Figs. 4 and 5). *E.coli*-produced recombinant cmvIL-10 was active in all the

experiments described above.

To determine whether cmvIL-10 is expressed by virus-infected cells, HEL 299 cells were infected with CMV strain AD169, 48 h after infection, analyses directed to this question were performed.

DNA and RNA were each isolated from virus-infected and control (uninfected) HEL 299 cells. Infection of the cells was confirmed by the presence of PCR-detectable CMV DNA, using CMV-specific primers and isolated DNA samples Figure 2B, lanes 4 and 5). The RNA samples were subjected to RT-PCR with sets of primers corresponding to those used for assessing the presence of CMV genome in infected cells (Figure 2B, lanes 6 and 7). Plasmids carrying the cmvIL-10, pEF-cmv_3 (Figure 2B, lane 2) or cmvIL-10 cDNA (spliced form, pEF-SPFL-cmvIL-10 (Figure 2B, lane 5) were used as positive controls.

The PCR product obtained with DNA from CMV-infected cells comigrated with the PCR product from control pEF-SPFL-cmvIL-10 plasmid (Figure 2B). RT-PCR with RNA from CMV-infected cells resulted in two products. The size of the smaller RT-PCR product was identical to that of the PCR product from the control pEF-SPFL-cmvIL-10 plasmid, whereas the size of the other RT-PCR product corresponded to the size of the PCR product from the control genomic construct, pEF-cmv_3. The larger RT-PCR product was derived from unspliced cmvIL-10 RNA, because PCR (without the RT step) with this RNA sample did not produce any products. No PCR or RT-PCR products were obtained with samples isolated from control uninfected cells.

RT-PCR also was performed with primers for β-actin cDNA to evaluate the integrity and quantity of the isolated RNA samples. PCR and RT-PCR fragments were isolated and sequenced. The sequence of the PCR product was identical to the sequence of the cmvIL-10 gene

(Figure 1); the sequence of the RT-PCR product revealed that both introns within the cmvIL-10 gene were spliced as they were spliced in COS cells transfected with pEF-cmv_2 plasmid.

Thus the cmvIL-10 gene is transcribed and the primary transcript is spliced to generate the cmvIL-10 mRNA (Figure 2B). In addition, cmvIL-10 is secreted by CMV-infected (Figure 6).

The conditioned media from the virus-infected and uninfected HEL 299 cells were assayed for the presence of IL-10 activity (Figure 6). The hamster cells expressing the chimeric IL-10R1/γR1 chain and the intact second chain, IL-10R2, as well as PBMC's, were used to perform the EMSA. Only medium from infected cells was able to induce Stat DNA binding complexes with the same pattern as IL-10 or cmvIL-10 treatment produced.

The amino acid sequence of cmvIL-10 can be considered in the context of both known and predicted structural features of IL-10. The crystal structure of cIL-10 revealed topological similarity to that of IFN-γ; both IL-10 and IFN-γ receptors belong to the same class II cytokine receptor family. Based on the structure of the IFN-γ:IFN-γR1 complex, the amino acid residues of IL-10 involved in its interaction with IL-10R1 were predicted (Fig. 3, asterisks). The crystal structure of ebvIL-10 has also been solved and is almost identical to that of IL-10. The fact that cmvIL-10 competes with cIL-10 for receptor binding indicates that the pattern of interaction of these proteins with receptor components is similar.

The cIL-10 protein has two intramolecular disulfide bridges (Fig. 3). Relative positions of all but one of the Cys residues are conserved. The cmvIL-10 protein has one additional Cys residue at position 78, which is apparently unpaired.

Assuming that the amino acid residues of cellular and viral IL-10s involved in receptor interaction are well conserved, the similarity between the primary sequences of cellular and viral IL-10s (ovIL-10, ebvIL-10, and cmvIL-10), allows validation of the model for IL-10:IL-10R1 interaction, and speculation about sites within IL-10 molecules involved in interaction with IL-10R2. Most of the residues within helices A and B and the AB-loop and helix F that were predicted to participate in interaction with IL-10R1 are well-conserved in all IL-10s (Fig. 3, bold asterisks). Several residues in these regions, however, as well as the last two residues at the C-terminus (Fig. 3, regular asterisks), were all predicted to interact with IL-10R1, but are not conserved; thus, it is likely they are not essential for interaction with IL-10R1, particularly because there are charge differences among these residues. These differences among the IL-10s may reflect differences in signal transduction and likely relate to subtle differences in their interactions with the receptor components. However, there are other conserved residues within helix A, the AB-loop, and helix F (Fig. 3, filled squares), suggesting they may be important either for interaction with IL-10R1 or for maintaining the structural integrity of the proteins. In addition, the existence of a few very well-conserved residues within helices C and D and the DE-loop (Fig. 3, open squares) in the middle part of the IL-10 sequences, the region which is apparently not involved in interaction with IL-10R1, suggests that these residues may be involved in interaction with IL-10R2, the second chain of the IL-10 receptor complex (Kotenko et al., 1997, supra).

It is interesting that ebvIL-10 shares many of the immunosuppressive activities but lacks several of the immunostimulatory activities of cIL-10. A single amino

acid, Ala at position 98 of ebvIL-10 (Fig. 3, filled triangle), accounts for these differences (Ding et al., 2000, J. Exp. Med, in press). This Ala is the only amino acid residue similar in all known IL-10 amino acid sequences except for that of ebvIL-10. Also, this Ala resides outside of the regions predicted to be involved in interaction with IL-10R1 and, thus, may be involved in interaction with IL-10R2.

Three regions of the CMV genome have been implicated in morphological transformation (Figure 7). However, the constant presence of only one of them, the morphological transforming region II (mtrII), was shown to be required in transformed cells to maintain the transformed phenotype (el-Beik et al., (1986) J. Virol. 60: 645-652). The mtrII was subsequently mapped to a minimal 980-base-pair *Ban*II/*Xho*I fragment located at the end of the unique long region (U_L) close to the inverted repeat (IR_L) (Fig. 7) (Razzaque et al., 1988, supra). The cmvIL-10-encoding region of the CMV genome, identified herein, is colinear with the mtrII region. Moreover, the interruption of a 79-amino acid ORF, the first region with calculated homology to cIL-10 (Figs. 1 and Fig. 7), abolished the transforming activity of the *Ban*II/*Xho*I fragment (Razzaque et al., 1988, supra; Jahan et al., (1989) J. Virol. 63: 2866-2869; Inamdar et al., (1992) Intervirology 34: 146-153; Chee et al., 1990, supra). Since disruption of the first exon of the cmvIL-10 gene also destroys the transforming ability of mtrII, a 79-amino acid polypeptide encoded by the reading frame colinear with the first exon of the cmvIL-10 gene could solely possess the tranforming activity, or oncongenic properties may be expressed by CMV-infected cells when the splicing of the cmvIL-10 mRNA is aberrant or incomplete.

To determine whether cmvIL-10 is indeed the protein responsible for mtrII-driven transformation,

investigation was conducted as to whether cmvIL-10 expressed in NIH3T3 cells can induce cell transformation. NIH3T3 cells were stably transfected with the pEF-cmv_3 plasmid. The pcDEF3 plasmid was used as a control. After 3 weeks of G418 selection, conditioned media from pools of transfectants were assayed for cmvIL-10 expression. Only medium from pEF-cmv_3-transfectants was positive in assays described earlier. To ascertain whether transfected cells are able to form tumors in syngeneic Swiss mice; three such Swiss mice each were injected subcutaneously with 1 x 10^6 cells transfected with either pEF-cmv_3 or pcDEF3 plasmids. After three weeks, two of the three mice injected with pEF-cmv_3-transfected cells developed tumors, but none of the control group (pcDEF3 transfected mice) had evidence of tumors.

Thus, although it remains to be conclusively proven that intact cmvIL-10 functions as an oncoprotein, given the results above and the known properties of IL-10s, the normal or abnormal processing and/or production of cmvIL-10 is a strong candidate as the mechanism by which CMV supports malignant tranformation.

Despite the limited homology between the cmvIL-10 and IL-10, cmvIL-10 binds to the IL-10 receptor complex, and competes with IL-10 for binding sites on the receptor (Figure 5). Further, cmvIL-10 is capable of inducing signal transduction events characteristic of IL-10 signaling and requires both chains of the IL-10 receptor complex to exert its biological activities (Figures 4 and 6). Thus CMV encodes its own novel, unique, functionally-active IL-10 homolog.

The present invention is not limited to the embodiments described and exemplified above, but is capable of variation and modification within the scope of the appended claims.

We claim:

1. An isolated nucleic acid molecule comprising a segment of a cytomegalovirus (CMV) genome located between nucleotides 159675 and 160376 of the CMV genome, the segment encoding an interleukin-10 (IL-10) protein.

2. A vector comprising the isolated nucleic acid molecule of claim 1.

3. The vector of claim 2, which is plasmid pEF-SPFL-cmv_2.

4. The nucleic acid molecule of claim 1, which encodes a polypeptide having a sequence selected from the group consisting of SEQ ID NO:2, a variant of SEQ ID NO:2 comprising a Thr residue inserted after Ala at position 26, and a variant of SEQ ID NO: 2 comprising a substitution of Thr for Ile at position 94.

5. The nucleic acid molecule of claim 4, comprising SEQ ID NO:1.

6. An isolated cmvIL-10 protein.

7. The cmvIL-10 protein of claim 6, produced by expression of a nucleic acid encoding the protein, inserted into a heterologous vector.

8. The cmvIL-10 protein of claim 6, having a sequence selected from the group consisting of SEQ ID NO:2, a variant of SEQ ID NO:2 comprising a Thr residue inserted after Ala at position 26, and a variant of SEQ

ID NO: 2 comprising a substitution of Thr for Ile at position 94.

9. A pharmaceutical formulation for treating a patient having a condition that responds to treatment with IL-10, which comprises a biological substance selected from the group consisting of a cmvIL-10 protein and a gene encoding a cmvIL-10 protein, in a pharmaceutically acceptable medium.

10. A method of treating a patient having an condition that responds to treatment with IL-10, which comprises administering to the patient an effective amount of the pharmaceutical formulation of claim 9 at a frequency and for a time sufficient to result in reduction or alleviation of the condition.

11. A method of treating a pathological condition associated with CMV infection, which comprises administering to a CMV-infected patient a composition capable of sequestering cmvIL-10 protein produced by the CMV in the infected patient, resulting in interference with the CMV life cycle, thereby reducing or eliminating the infection.

12. A method for detecting CMV in a sample isolated from a patient, comprising:

a) obtaining the sample from the patient;

b) contacting the sample with antibodies immunologically specific for one or more epitopes of a cmvIL-10 protein;

c) detecting the formation antibody:antigen complexes, the presence and amount of the complexes being indicative of the presence and amount of CMV in the patient.

13. A kit for detecting or purifying a cmvIL-10 protein or a *cmvIL-10* nucleic acid molecule, which comprises:

a) one or more of a reagent selected from the group consisting of: antibodies immunologically specific for a cmvIL-10 protein and nucleic acid molecules that specifically hybridize with *cmvIL-10* nucleic acid molecules;

b) instructions for using the antibodies or the nucleic acid molecules to detect or purify the cmvIL-10 protein or the *cmvIL-10* nucleic acid molecules; and, optionally,

c) one or more reagents for using the antibodies or the nucleic acid molecules to detect or purify the cmvIL-10 protein or the *cmvIL-10* nucleic acid molecules.

```
    1       MHSSALLCCLVLLTGVRASPGQGTQSENSCTHFPGNLPNMLRDLRDAFSRVK 52      IL-10
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159678 MLSVMVSSSLVLIVFFLGASEEAKPATTTIKNTKPQCRPEDYATRLQDLRVTFHRVK 159848 The CMV genome

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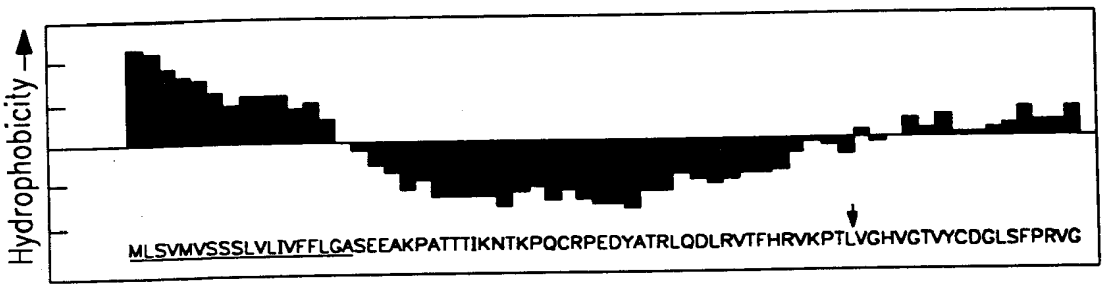
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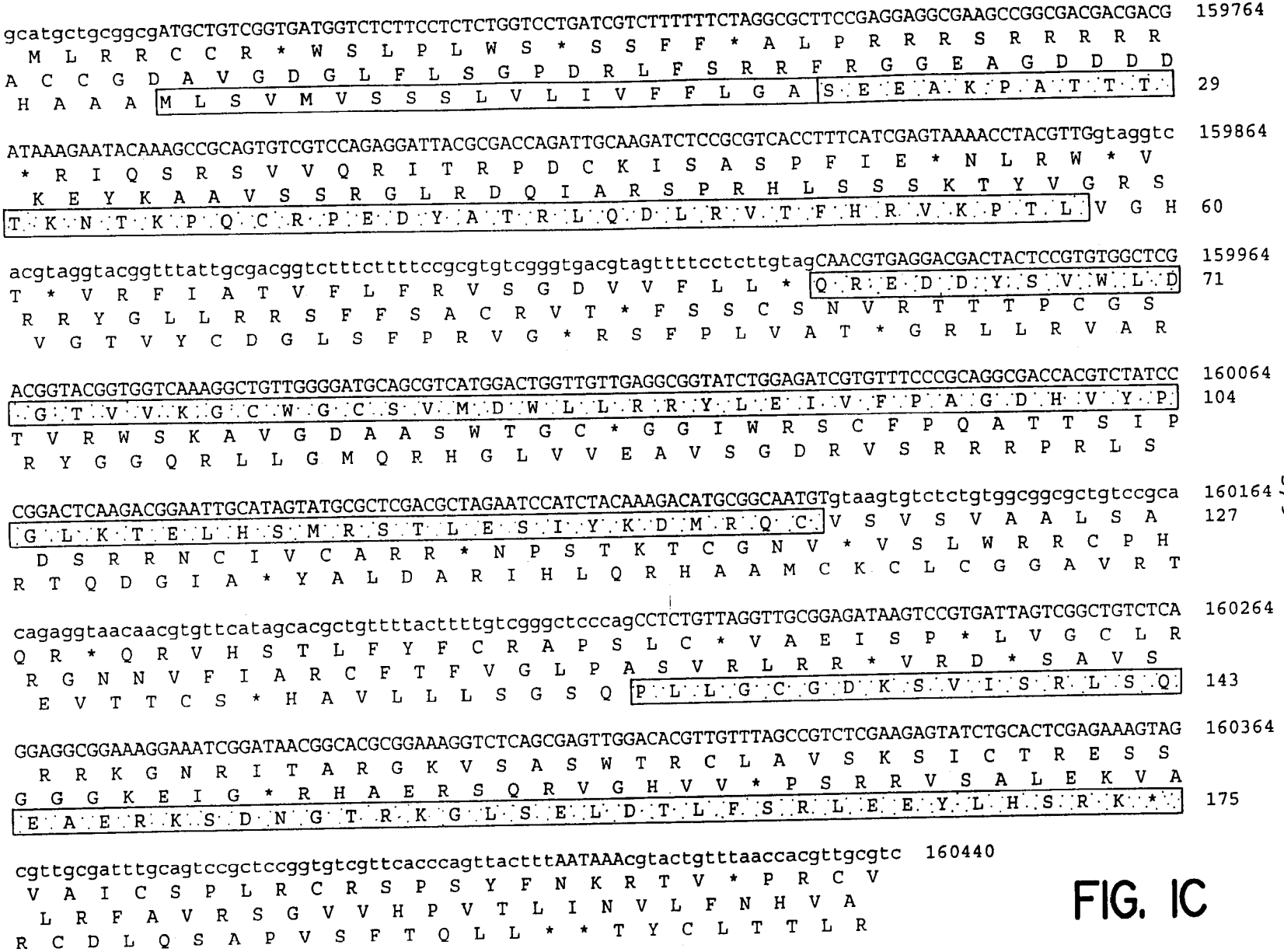

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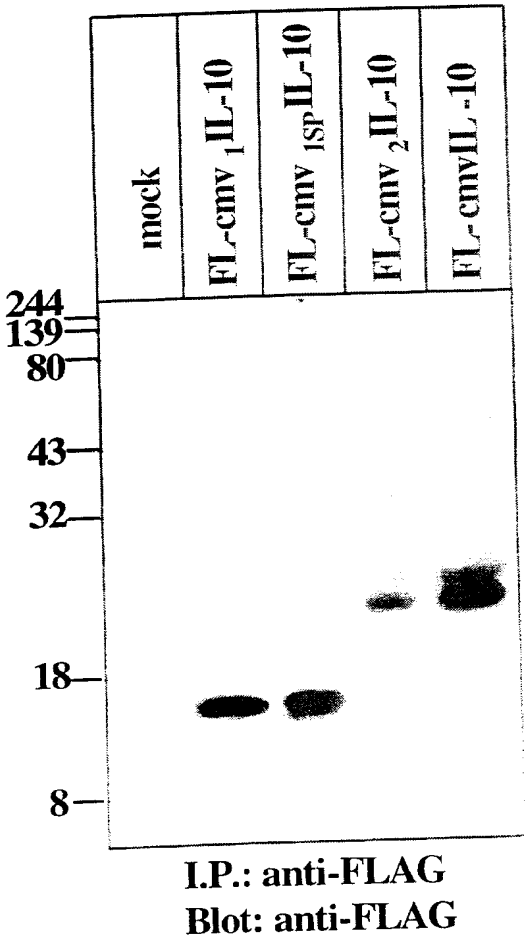
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FL-cmv 1SPIL-10
FL-cmv 2IL-10
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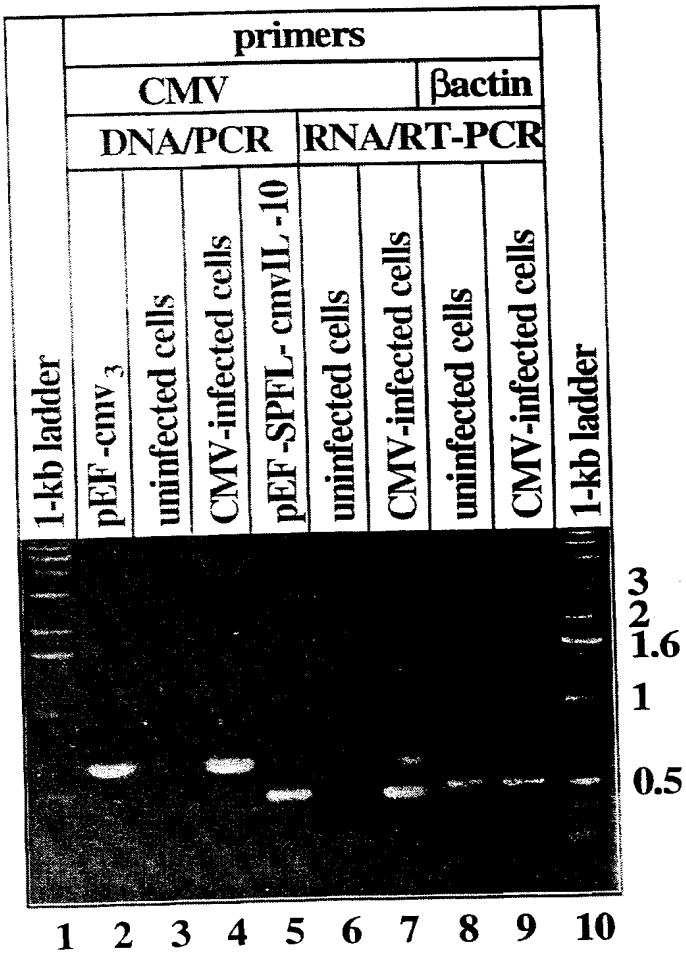
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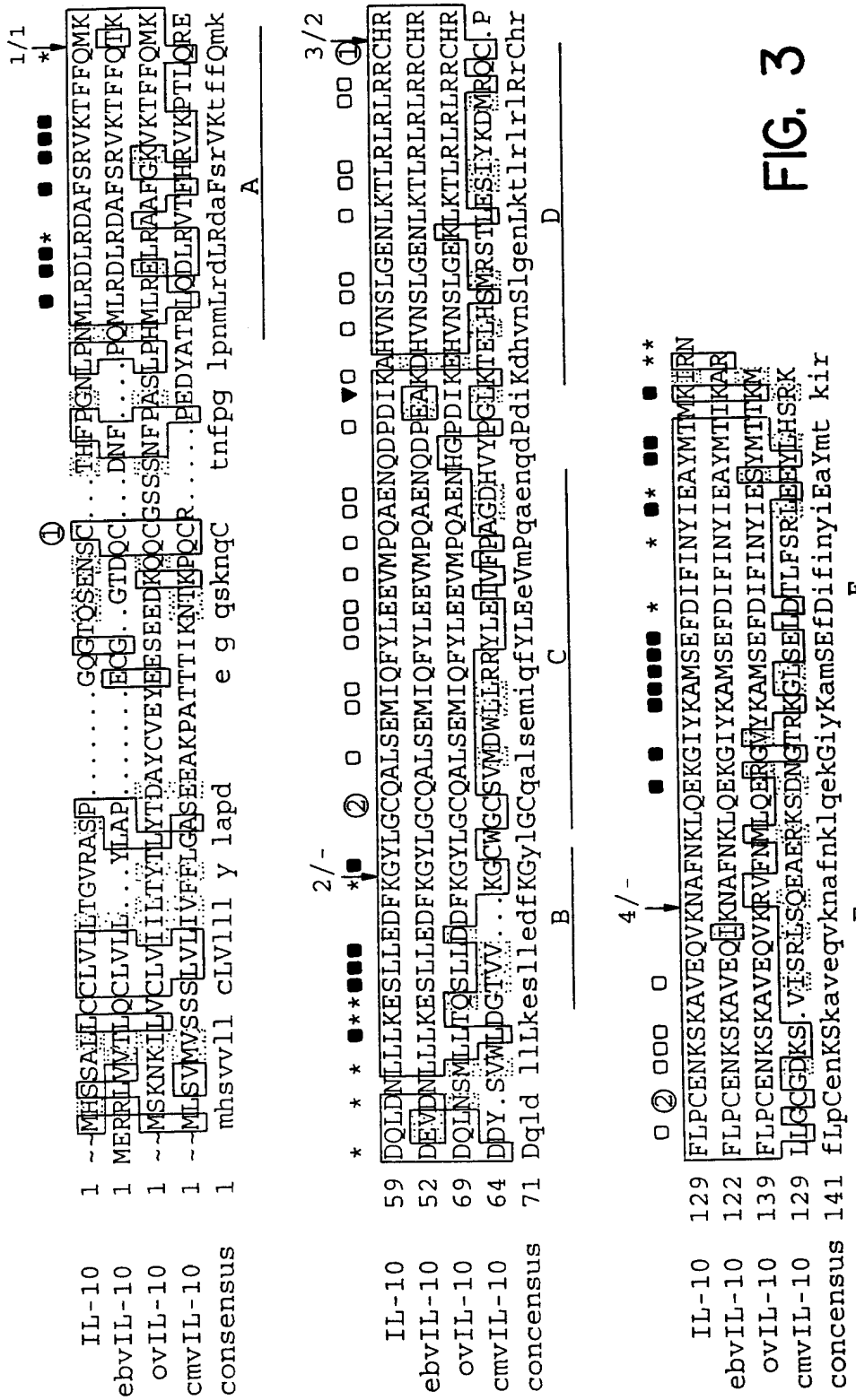
FIG. 2B



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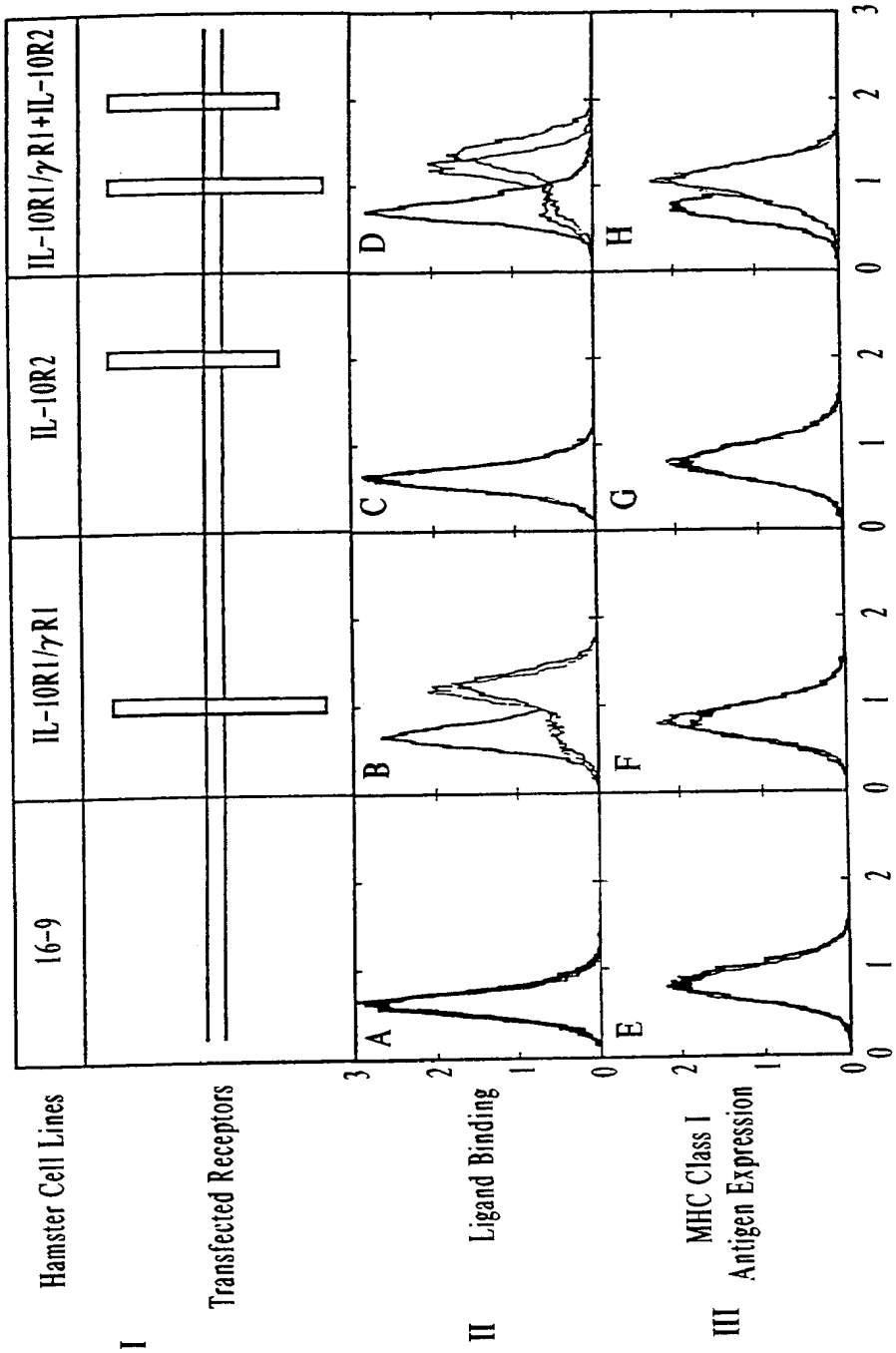
16-9
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IL-10R2
IL-10R1/γR1+IL-10R2
I Hamster Cell Lines
Transfected Receptors
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III MHC Class I Antigen Expression
A
B
C
D
E
F
G
H
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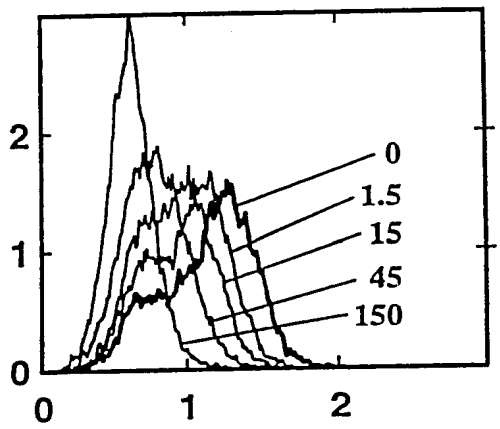
1
2
3

FIG. 4



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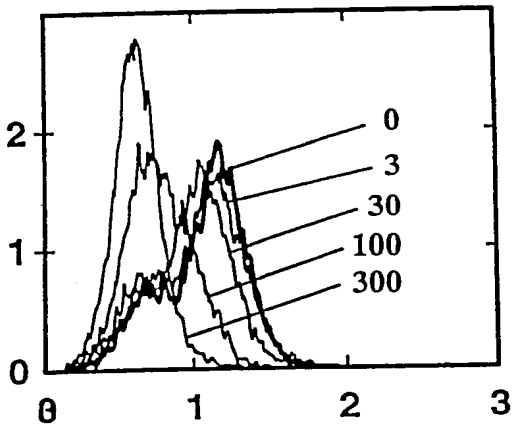

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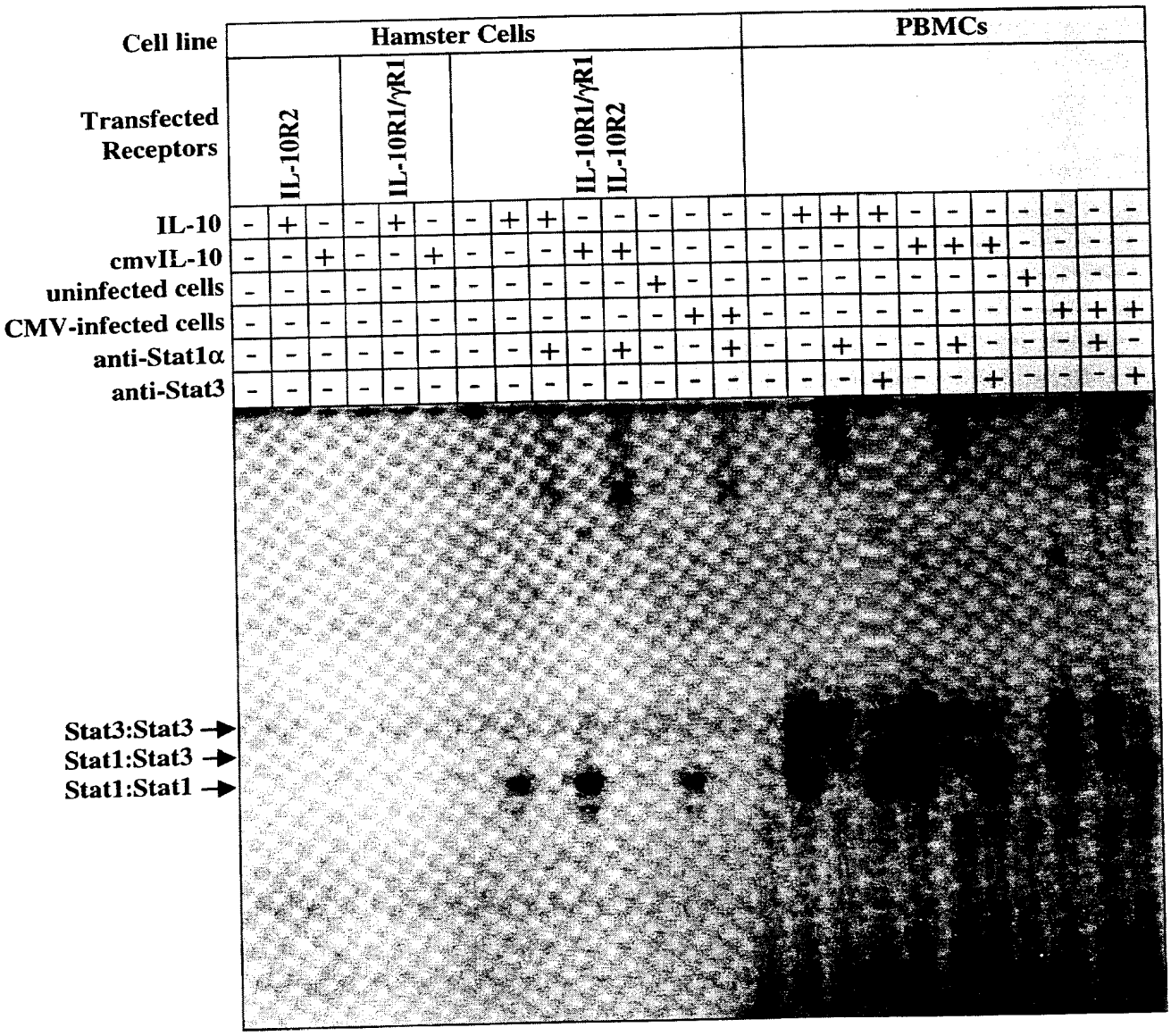
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Stat1:Stat1

FIG. 6

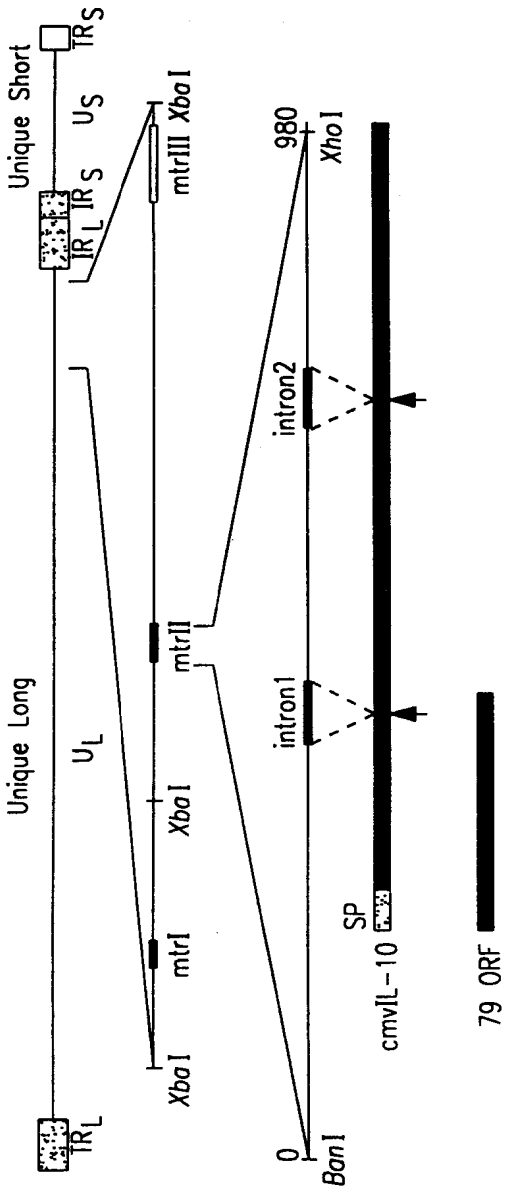
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FIG. 7

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Pestka, Sidney

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/24213

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C07H 21/02, 21/04
US CL :536/23.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	RAZZAQUE. A. Localization and DNA sequence analysis of the transfo rming domain (mtrII) of human cytomegalovirus. Proc. Natl. Acad. Sci. August 1988, Vol. 85, pages 5709-5713 see entire document.	6-8 ----- 9-13

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 20 OCTOBER 2000	Date of mailing of the international search report 22 JAN 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer BRETT L NELSON Telephone No. (703) 308- TERRY J. DEY PARALEGAL SPECIALIST TECHNOLOGY CENTER 1600

Form PCT/ISA/210 (second sheet) (July 1998) ★

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

WEST, DIALOG, MEDLINE, SCISEARCH, BIOSIS, EMBASE

search terms: cytomegalovirus, IL-10, CMV, DNA, protein, amino aicd, polypeptide, antibodies, diagnositic, pharmaceutical, treatment